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Intermedilysin, a Novel Cytotoxin Specific for Human Cells, Secreted by *Streptococcus intermedius* UNS46 Isolated from a Human Liver Abscess

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A novel cytotoxin (intermedilysin) specific for human cells was identified as a cytolytic factor of *Streptococcus intermedius* UNS46 isolated from a human liver abscess. Intermedilysin caused human cell death with membrane blebs. Intermedilysin was purified from UNS46 culture medium by means of gel filtration and hydrophobic chromatography. The purified toxin was resolved into major and minor bands of 54 and 53 kDa, respectively, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These proteins reacted with an antibody against intermedilysin. Five internal peptide fragments of intermedilysin were sequenced and found to have 42 to 71% homology with the thiol-activated cytotoxin pneumolysin. However, the action of intermedilysin differed from that of thiol-activated cytotoxins, especially in terms of a lack of activation by dithiothreitol and resistance to treatments with *N*-ethylmaleimide and 5,5′-dithio-bis-(2-nitrobenzoic acid), although cholesterol inhibited the toxin activity. Intermedilysin was potently hemolytic on human erythrocytes but was 100-fold less effective on chimpanzee and cynomolgus monkey erythrocytes. Intermedilysin was not hemolytic in nine other animal species tested. Since human erythrocytes treated with trypsin were far less sensitive to intermedilysin than were the intact cells, a cell membrane protein(s) may participate in the intermedilysin action. These data demonstrated that intermedilysin is distinguishable from all known bacterial cytolysins.

Streptococcus intermedius is one of the *Streptococcus milleri* group of streptococci (SMG). The SMG constitute a portion of the normal flora of the human oral cavity, as well as the upper respiratory, gastrointestinal, and female urogenital tracts, and it can cause purulent infections in the mouth and internal organs, including the brain, liver, lungs, and spleen (3, 8, 9, 19, 22, 26–28, 33, 38). Three distinct species within the SMG (*S. anginosus*, *S. constellatus*, and *S. intermedius*) are recognized, and their association with body sites and clinical conditions has been determined (30, 41, 43, 44). *S. intermedius* is associated with brain and liver abscesses (42). Various proteins and enzymes, such as immunosuppressive protein P90, serum albumin-binding protein, glycosidases, and hemagglutinating factor, are thought to be involved in purulent abscess formation by this species (2, 4, 13, 14, 45, 47). However, the pathogenic factors of *S. intermedius* have not been characterized despite the acknowledged clinical importance of the SMG and this species (6, 12, 31).

Several *Streptococcus* species are hemolytic (alpha- or beta-) on blood agar. These species include *S. pyogenes*, *S. pneumoniae*, and *S. suis*, which produce the cytotoxins streptolysin O (18), pneumolysin (40), and suilysin (16), respectively. These cytotoxins directly damage host tissues and defense cells and participate in bacterial pathogenicity (34). These cytotoxins possess a conserved 11-mer sequence containing a cysteine residue near their C termini (5). Reducing the cytotoxins with a thiol compound such as dithiothreitol (DTT) activates their cytolytic actions. However, the structural changes induced by the oxidation or alkylation of the cysteine residue or substitution of the tryptophan residue with an alanine residue in the conserved sequence inactivates the toxins. Thus, they are called thiol-activated cytotoxins, and the integrity of the steric structure around the conserved sequence is thought to be essential for inducing host cell cytolysis. These cytotoxins are effective on a variety of animal cells, such as sheep, rabbit, and horse erythrocytes, as well as on human cells (34). However, since almost all strains of *S. intermedius* do not induce significant hemolysis on the horse or sheep blood agar ordinarily used in clinical microbiology (35, 44), neither the cytotoxin nor the hemolysin of this species has been studied. During this investigation, we found a unique cytolytic toxin (intermedilysin) secreted from a clinical strain of this species isolated from a human liver abscess. We purified and characterized intermedilysin and found that its action is human cell specific, a feature that has never been found with bacterial cytolytic toxins.

MATERIALS AND METHODS

Bacteria and animal cell lines. *S. intermedius* UNS46 from a human liver abscess (41) was maintained on Todd-Hewitt broth-based 5% (vol/vol) sheep blood agar at 37°C in the presence of 5% (vol/vol) CO_2. The human hepatoma cell line HepG2 was a gift from N. Katunuma (Institute for Health Science, Tokushima Bunri University, Tokushima, Japan). JM and NB69, a human T-cell leukemia and a human neuroblastoma, respectively, were purchased from the Riken Cell Bank (Tsukuba, Japan). HepG2, JM, and NB69 cells were cultured at 37°C in the presence of 5% (vol/vol) CO_2 in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum (FCS), RPMI 1640 medium containing 10% (vol/vol) FCS, and RPMI 1640 medium containing 15% (vol/vol) FCS, respectively. The FCS was heat inactivated at 56°C for 30 min before use.

Purification of intermedilysin from culture supernatant of *S. intermedius* UNS46. Strain UNS46 was cultured for 18 h at 37°C in a medium consisting of a 1:4 mixture of brain heart infusion broth (Difco Laboratories) and the chemically defined FMC broth medium (36) supplemented with 0.5% (wt/vol) glucose.

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The supernatant was collected by centrifugation and then lyophilized. Twenty grams of the lyophilized powder was dissolved in 40 ml of Milli-Q water and dialyzed against 2 liters of Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) for 3 h four times at 4°C. The crude dialysate was concentrated by ultrafiltration using an Amicon YM-10 membrane to bring the volume of 20 ml at 4°C. This sample then was applied to an Ultrogel AcA34 column (4 cm in diameter and 87 cm long; IBF Biotechnics, Villeneuve la Garenne, France) and eluted in 150 mM NaCl containing 10 mM potassium phosphate (pH 7.0). Subsequently, 20 μl of each fraction was assayed for cytotoxicity (JM cytotoxicity assay) as described below. Active fractions were combined and concentrated to 1.5 to 2.5 ml by ultrafiltration using an Amicon YM-10 membrane at 4°C. An equal volume of 50 mM sodium phosphate buffer containing 3 M ammonium sulfate (pH 7.0) was mixed with the concentrated fraction. The mixture was then further purified by high-performance liquid chromatography on a phenyl-Superose HR 5/5 column (Pharmacia-Biotech, Uppsala, Sweden) equilibrated with 50 mM sodium phosphate buffer containing 1.5 M ammonium sulfate (pH 7.0). The sample was eluted with a two-step descending linear gradient of ammonium sulfate from 1.5 to 0 M. The cytolytic activity of 20 μl of the peak and the shoulder fractions was determined in the JM cytotoxicity assay. Active fractions were combined and concentrated by ultrafiltration as described above. An equal volume of 50 mM sodium phosphate buffer containing 3 M ammonium sulfate (pH 7.0) was mixed with the condensed fractions. The mixture was again resolved by phenyl-Superose chromatography in the manner described above. Fractions that contained cytotoxic activity were combined, concentrated, and frozen at −85°C until use.

Partial primary sequencing of intermedilysin. Purified intermedilysin was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transblotted onto a polyvinylidene fluoride membrane, and stained with 0.5% (wt/vol) Ponceau S in 1% (vol/vol) acetic acid. Both bands were simultaneously excised from the membranes; then the toxin was cleaved with 1 μg of lysylendopeptidase at 37°C for 16 h. The reaction mixture was then subjected to reverse-phase chromatography (Waters Bondasphere C_{18} column; 2.1 mm in diameter and 650 mm long; Waters, Milford, Mass.) with a 0 to 60% (vol/vol) acetonitrile ascending linear gradient in the presence of 0.1% (vol/vol) trifluoroacetic acid. The primary sequences of the five separated peptides, 54KL2, 54KL7, 54KL8, 54KL11, and 54KL14, were analyzed on an Applied Biosystems 477A protein sequencer (Applied Biosystems, Foster City, Calif.).

Assay of cytolytic effect on nucleated cells. To trace the cytolytic activity in fractions obtained during purification, 80 μl of JM cell cultures in the late logarithmic phase (5×10^5 to 10×10^5 cells per ml; viability, >90%) and 20 μl of the preparation containing intermedilysin were mixed in a microcentrifuge tubes and incubated at 37°C in the presence of 5% (vol/vol) CO_2. To estimate the specific cytolytic activity, 80 μl of JM cell suspension in PBS was used instead of the cell culture. After a 1-h incubation, 100 μl of PBS containing 0.4% (wt/vol) trypan blue was added, and the mixture was transferred to a hemocytometer. The cytolytic effect (cytotoxicity) was estimated by means of trypan blue exclusion as follows: cytotoxicity (%) = [(number of trypan blue-stained $cells_{exp1}$/number of total $cells_{exp1}$)−(number of trypan blue-stained $cells_{exp2}$/number of total $cells_{exp2}$)] × 100. The suffixes exp1 and exp2 refer to the results obtained when 20 μl of an unknown test sample was assayed and when 20 μl of PBS was used in the assay (negative control), respectively. To examine the effects of trypsin and heat on the cytolytic activity of intermedilysin, the activities of the 1-mg/ml trypsin-treated and 100°C-treated samples were also observed. For morphological observations, human cells (HepG2, NB69, and JM) were incubated in the corresponding culture medium containing intermedilysin at 37°C for 1 h in the presence of 5% (vol/vol) CO_2. Morphological changes were examined by means of phase-contrast microscopy.

Hemolysis assay. Human blood was obtained from healthy Japanese volunteers, and blood samples from cynomolgus monkeys (*Macaca fascicularis*) and chimpanzees (*Pan troglodytes*) were gifts from the Taiho Pharmaceutical Co., Ltd. (Tokushima, Japan) and The Green Cross Co. (Kanzaki, Japan), respectively. Chicken (*Gallus gallus* var. *domesticus*), cow (*Bos taurus*), horse (*Equus caballus*), and sheep (*Ovis aries*) blood was purchased from the Nippon Bio-Supply Center (Tokyo, Japan). Cat (*Felis catus*), dog (*Canis familiaris*), rabbit (*Oryctolagus cuniculus* var. *domesticus*; Japanese white rabbit), rat (*Rattus norvegicus*; SD), and mouse (*Mus musculus*; BALB/c) blood was obtained from healthy normal animals. All blood cells were stored in sterilized Alsever solution for 1 to 3 days at 4°C until use. Erythrocytes were washed with PBS three times at 4°C by centrifugation using a Hitachi model 05PR-22 centrifuge (3,000 rpm for 10 min in a 05 swing-out rotor; Hitachi, Tokyo, Japan) before use. Chilled PBS containing 5×10^7 erythrocytes per ml and the amounts of the intermedilysin fraction indicated in the figure legends were mixed in microcentrifuge tubes (total of 1 ml) in an ice-water bath, and then hemolysis proceeded at 37°C for 1 h. After the reaction, the mixture was centrifuged at 2,500 rpm in a Kubota model 1900 microcentrifuge (with an RA-53 fixed-angle rotor; Kubota, Tokyo, Japan) at 4°C for 5 min. The A_{540} of the supernatant was measured in a Hitachi model U-2000 spectrophotometer. Percent hemolysis was calculated as follows: hemolysis (%) = [(absorbance of supernatant from the sample containing intermedilysin − absorbance of supernatant from the sample containing no intermedilysin)/(absorbance of supernatant from the sample completely hemolyzed by hypotonic processing − absorbance of supernatant from the sample containing no intermedilysin)] × 100. The index of hemolytic activity, HD_{50}, was the concentration of intermedilysin giving 50% hemolysis in the assay described above. Human erythrocytes were used in all experiments except those to evaluate the species specificity of intermedilysin action. We confirmed that the hemolysis induced by the effectors, pH drift (±0.1) from 7.4, or the incubation temperature was negligible. Other conditions were as specified for each figure and table.

The hemolysis induced by the bacterial cells was examined on Todd-Hewitt broth-based blood agar containing 5% (vol/vol) defibrinogenized sheep, horse, rabbit, and human blood after an incubation at 37°C for 2 days in an atmosphere 5% (vol/vol) CO_2.

Antibody. Antibody against a synthetic peptide possessing the partial sequence of intermedilysin (N-terminal part of 54KL11; GSNFSAQSPAVPIS) was raised in a rabbit. The 54KL11 peptide was synthesized as a multiple-antigen peptide (MAP) with eight identical peptide branches, using 9-fluorenylmethoxycarbonyl chemistry in a Shimadzu model PSSM-8 peptide synthesizer (Shimadzu, Kyoto, Japan) as reported previously (29). PBS containing 1 mg of MAP was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into the back of the rabbit. Thereafter, two booster shots were given at 1-week intervals, using Freund's incomplete adjuvant and 0.5 mg of antigen. The anti-54KL11-MAP immunoglobulin G (IgG) was prepared by using protein A affinity chromatography from the antiserum which showed high titer in an enzyme immunoassay as described previously (25). Anti-streptolysin O rabbit serum was the product of Nissui (Tokyo, Japan; catalog no. 07003). IgG purified from normal preimmune rabbit serum was used as a negative control.

Electrophoresis and immunoblotting. SDS-PAGE was performed with 10% separating gels as described by Laemmli (21). The gel was stained with silver, using a kit from Wako Pure Chemicals (Osaka, Japan). To immunoblot intermedilysin, 0.5 μg of the toxin separated by SDS-PAGE was electroblotted onto a polyvinylidene fluoride membrane (Durapore; Nihon-Millipore, Yonezawa, Japan) by the method of Kyhse-Andersen (20). Each lane of the blotted membrane was excised and immunostained with anti-54KL11-MAP rabbit IgG or preimmune IgG as the first antibody and anti-rabbit IgG antibody labeled with alkaline phosphatase as the second antibody as described previously (25).

Total protein assay. Total protein was estimated by the bicinchonic acid method, using a bicinchoninic acid protein assay kit purchased from Pierce (Rockford, Ill.). Bovine serum albumin (BSA) was used as the standard protein.

RESULTS

Cytolytic effect of intermedilysin secreted from *S. intermedius* UNS46 on human cells. Following the addition of UNS46 culture medium, blebs formed from several sites on the surface of human cells (NB69, HepG2, and JM) within 5 to 10 min. Finally, the cytosol became completely separated from the organelles as a large bleb (Fig. 1, upper panel, 1 and 2). This cytolytic action was prevented by first heating the bacterial components at 100°C for 5 min (Fig. 1, upper panel, 3) or by digesting them with 1 mg of trypsin per ml at 37°C for 1 h (data not shown). Subsequently, hemolysis around the colonies of the bacteria on blood agar plates containing sheep, horse, rabbit, or human erythrocytes was determined (Fig. 1, lower panel). Strain UNS46 caused complete hemolysis (beta-hemolysis) only on agar containing human blood. These results suggested that the cytolytic toxin secreted from the bacterial cells is a protein and that it is specifically cytotoxic to human cells. The cytolytic toxin was subsequently referred to as intermedilysin.

Purification of intermedilysin. The rate of growth of strain UNS46 increased threefold in the medium consisting of 1 volume of brain heart infusion medium and 4 volumes of FMC medium supplemented 0.5% (wt/vol) glucose compared with that in brain heart infusion alone containing 0.5% (wt/vol) glucose. Since we also confirmed the cytolytic activity in the lyophilized UNS46 culture medium (Fig. 1), we purified intermedilysin by using lyophilized cultured medium as the starting material. To determine the cytolytic activity in the chromatography fractions, cell line JM in late log phase was used as the target, since membrane damage was easily estimated in the suspended cells by trypan blue exclusion. Moreover, the results obtained from the assay using a condition-controlled single cell line were reproducible for every preparation.

Figure 2A shows the results of Ultrogel AcA34 gel filtration chromatography. The peak of JM cytolytic activity was recovered in the fractions with a molecular mass of about 50 kDa.

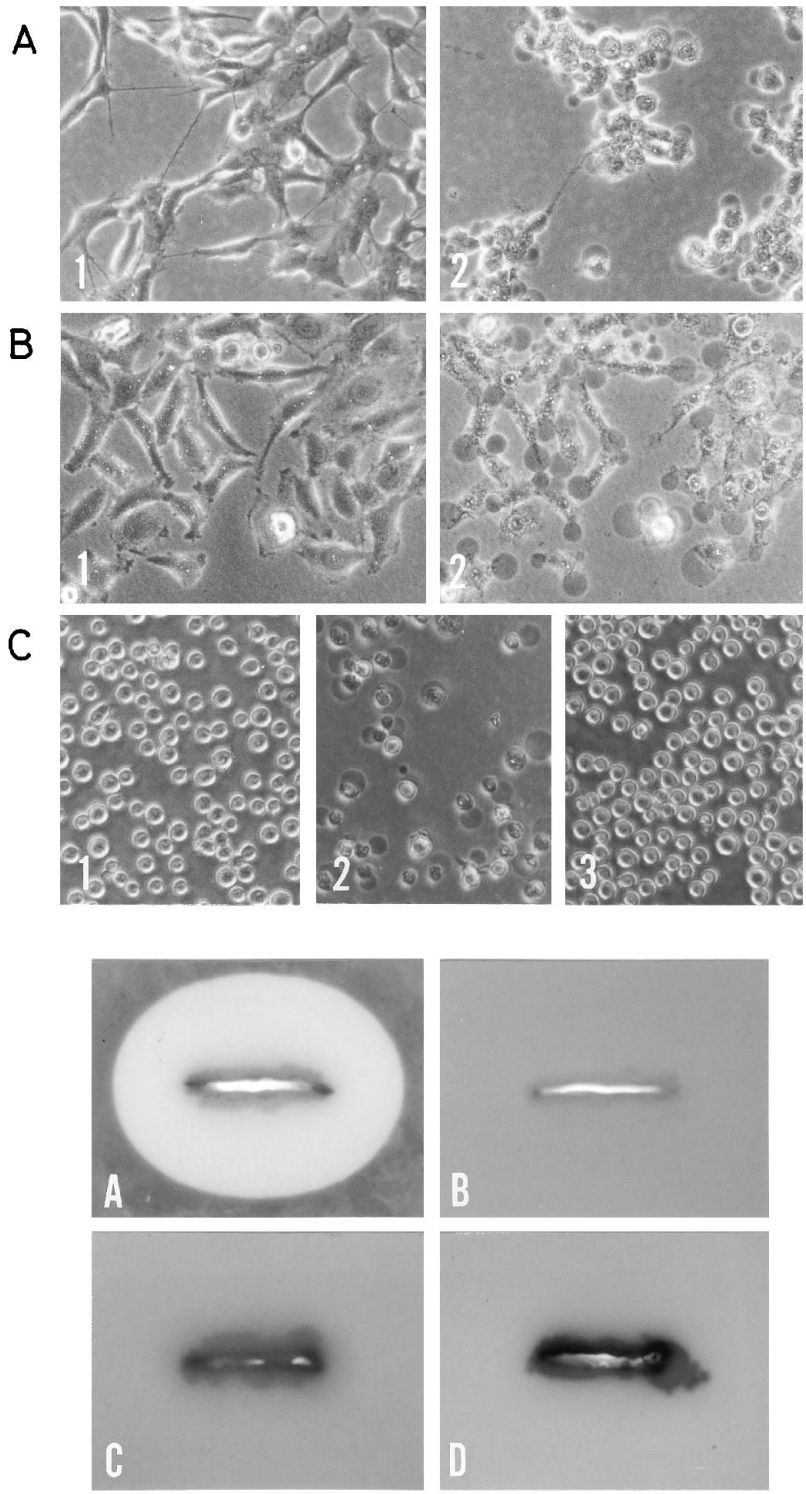

FIG. 1. Secretion of intermedilysin from the *S. intermedius* UNS46. In the upper panel, NB69 (A) and HepG2 (B) cells at 50% confluence on a gelatin-coated plate (diameter, 6 cm) and JM (C) cells in late log phase were incubated in the culture medium appropriate to each cell line containing 0.25 mg of lyophilized UNS46 culture medium per ml at 37°C for 1 h in the presence of 5% (vol/vol) CO_2. 1, before the incubation; 2, after incubation with native UNS46 culture medium components; 3, after incubation with the UNS46 culture medium components heated at 100°C. In the lower panel, *S. intermedius* UNS46 was inoculated onto agar containing human (A), sheep (B), horse (C), and rabbit (D) blood by puncturing with a loop and then incubated at 37°C for 2 days in the presence of 5% (vol/vol) CO_2. The results of one of two experiments are shown.

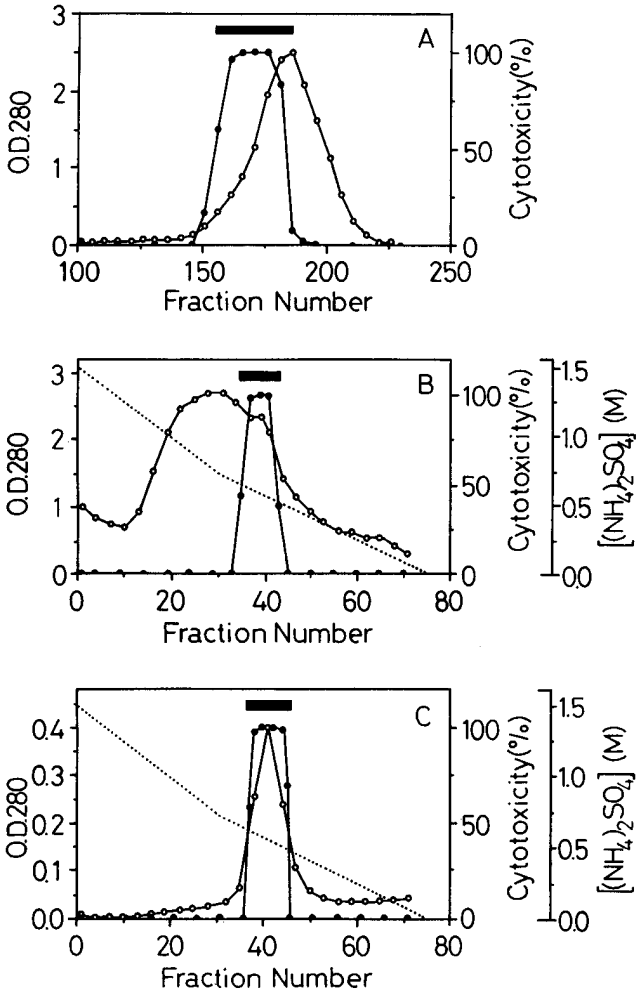

FIG. 2. Purification of intermedilysin by gel filtration and hydrophobic chromatography. (A) Ultrogel AcA34 gel filtration chromatography of the concentrated UNS46 culture medium. Fraction size, 6 ml. (B) Phenyl-Superose HR5/5 hydrophobic chromatography of the active fractions obtained from the Ultrogel step. Fraction size, 0.5 ml. (C) Second phenyl-Superose HR5/5 hydrophobic chromatography of the active fractions obtained in the second step. Open circles, optical density at 280 nm (O.D. 280); filled circles, JM cytotoxic activity; filled boxes, active fractions containing intermedilysin. Dotted lines show the two-step linear ammonium sulfate gradient.

The active fractions were combined and condensed by ultrafiltration. The concentrated active fraction was then purified by hydrophobic chromatography on a phenyl-Superose column. The cytolytic activity eluted in the range of 480 to 600 mM ammonium sulfate (Fig. 2B). After the first hydrophobic chromatography, the active fractions were combined and dialyzed against PBS, and the procedure was repeated. A single cytolytic activity peak and a single protein peak appeared within the same range of ammonium sulfate as described above (Fig. 2C). The cytolytic activity was susceptible to heat or trypsin throughout the purification.

The SDS-PAGE profile of the sample at each purification step is shown in Fig. 3. The purified intermedilysin fraction contained a major 54-kDa and a minor 53-kDa band (Fig. 3A). Both bands were detected in samples exposed or not exposed to β-mercaptoethanol (Fig. 3B). The staining profile was not affected by thiol, suggesting that these bands are distinct molecules and that the doublet was not due to polymorphism induced by intramolecular disulfide formation.

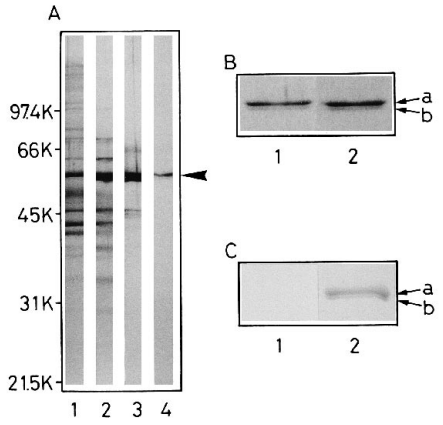

FIG. 3. SDS-PAGE of the intermedilysin fraction at each purification step and immunoblotting of purified intermedilysin. (A) Determination of the purity of the intermedilysin fraction obtained at each step shown in Fig. 2 by silver staining after SDS-PAGE (10 μl per lane). Lane 1, concentrated crude UNS46 culture medium dialyzed and ultrafiltrated; lane 2, concentrated active fraction after Ultrogel AcA34 gel filtration; lane 3, combined active fraction after the first phenyl-Superose HR5/5 hydrophobic chromatography; lane 4, combined active fraction after a repeat hydrophobic chromatography (purified intermedilysin). The arrowhead points to intermedilysin. (B) Silver staining image after the SDS-PAGE of the purified intermedilysin in the solubilizing buffer for SDS-PAGE in the presence (lane 1) or absence (lane 2) of 512 mM β-mercaptoethanol. (C) Immunoblotting of purified intermedilysin. Lane 1, preimmune IgG; lane 2, anti-54KL11-MAP IgG. In panels B and C, arrows a and b show 54- and 53-kDa bands, respectively. The results of one of two experiments are shown.

The purification of intermedilysin is summarized in Table 1. The cytolytic activity of the active fraction at each step of the purification was estimated by the hemolytic reaction using human erythrocytes as the target cells. After chromatography, intermedilysin was purified 351-fold from the reconstituted and concentrated UNS46 culture medium. The index of the cytolytic activity (HD_{50}) of purified intermedilysin was 0.65 ng, which is comparable with that of other cytotoxins (34). The lytic effect of intermedilysin on JM cells in PBS was similar to that on human erythrocytes (data not shown).

Partial primary sequence of intermedilysin. After SDS-PAGE and membrane transfer, we attempted to sequence the amino acids of the N-termini of the intermedilysin bands. However, no sequence information was obtained, perhaps because the N termini of both bands were masked. We therefore determined the internal sequences of intermedilysin. Fragments of the toxin cleaved by lysylendopeptidase were recovered by reverse-phase chromatography. The primary sequences of the five fragments are shown in Fig. 4. Among them, the sequence of the heptapeptide 54KL2 (FGADFSK) was highly homologous (71%) to that of pneumolysin. However, the other peptides, 54KL7, 54KL8, 54KL11, and 54KL14, possessed only 40, 47, 58, and 42% homology with pneumolysin, respectively. The primary sequence of the 54KL11 fragment was relatively conserved in pneumolysin together with other thiol-activated cytotoxins, such as streptolysin O, perfringolysin O, alveolysin, and listeriolysin O, with which it had 53, 53, 53, and 47% homology, respectively (11, 18, 23, 37, 40). We raised polyclonal antibodies against a synthetic peptide representing the sequence of the 54KL11 fragment. Western blots (immunoblots) of the purified intermedilysin fraction reacted with the antibody are shown in Fig. 3C. The 54- and 53-kDa bands were both stained by the antibody, indicating that they carry the 54KL11 sequence.

Time course of the cytolytic action of intermedilysin. After a lag of about 2 min, hemolysis proceeded rapidly over the next

TABLE 1. Summary of the purification of intermedilysin

Step	Total protein[a] (mg)	Total activity[a] (U)	Sp act[a] (U/mg)	Yield (%)	Purification (fold)
Ultrafiltration	2.61×10^2	1.15×10^6	4.41×10^3	100	1.0
Ultrogel AcA34	2.24×10	1.10×10^6	4.91×10^4	96	11.1
Phenyl-Superose HR5/5	3.50×10^{-1}	4.51×10^5	1.29×10^6	39	293
Phenyl-Superose HR5/5 rechromatography	1.10×10^{-1}	1.71×10^5	1.55×10^6	15	351

[a] Average of duplicate determinations which did not differ by more than 5%. The cytolytic activity of intermedilysin was estimated by using human erythrocytes of blood type AB. U, the activity showing 50% hemolysis in the standard hemolysis assay described in Materials and Methods. These values are those of the preparations shown in Fig. 2.

10 min. About 80% of erythrocytes were hemolyzed within the first 10 min of the incubation with intermedilysin. Hemolysis was complete 60 min later.

Target cell specificity of intermedilysin. The unique feature of intermedilysin was its species-specific cytolysis. Figure 5 shows a typical result of the action of intermedilysin on various animal erythrocytes. Intermedilysin acted as a potent cytolytic toxin on human erythrocytes, whereas it was about 100-fold less effective on those of other primates, such as chimpanzees and cynomolgus monkeys. The HD_{50} values (means ± standard deviations) for five humans, chimpanzees, and cynomolgus monkeys were 0.8 ± 0.2, 110 ± 33, and 91 ± 15 ng/ml, respectively. The susceptibility of human erythrocytes to intermedilysin was not dependent on the ABO blood type of the cells. Intermedilysin had no lytic effects on cat, chicken, cow, dog, horse, mouse, rabbit, rat, and sheep erythrocytes, even when the toxin concentration was increased 5,000-fold over that giving an HD_{50} with human erythrocytes. These results show that the cytolytic action of intermedilysin was specific for primates, especially humans.

The pH dependence of intermedilysin stability. After 37°C incubation for 1 h of intermedilysin in the various pH buffers containing 150 mM NaCl and 10 mM buffering reagent, the toxin solution was diluted 8,000-fold in PBS, and the remaining hemolytic activity was assayed. Intermedilysin was the most stable in the physiological pH range (6 to 8), particularly at 7.0. At lower and higher pH values, the activity was gradually lost. However, 50% of the full cytolytic activity remained even at pH 4.0.

Temperature dependence of the stability and reactivity of intermedilysin. The effect of temperature on the stability of intermedilysin and the temperature dependence of its cytolytic reaction are shown in Fig. 6. Intermedilysin was relatively stable between 0 and 30°C, although the toxic activity gradually decreased with an increase in the initial incubation temperature (Fig. 6A). Above 40°C, the activity of intermedilysin began to decrease remarkably, and it was abolished at temperatures above 50°C. The cytolytic reaction was not detected at 0°C (ice-water incubation). However, significant toxin activity was measured at temperatures above 10°C, especially at the physiological temperature range of the host (Fig. 6B). The reaction was saturated at 30°C and then decreased slightly upon further increases in the reaction temperature, possibly because intermedilysin is unstable at high temperatures. The half-maximal reaction temperature was about 7°C.

Effects of salts on the stability of intermedilysin. Toxin activity was lost after ion-exchange chromatography in a low ion concentration buffer. We therefore examined the effect of salt on the stability of intermedilysin. The toxin was incubated with salts in 10 mM sodium phosphate buffer (pH 7.4) at 37°C for 1 h, diluted 8,000-fold in PBS, and subjected to the hemolysis assay. Both the concentration and the ion species of the salt in the buffer significantly affected the stability of intermedilysin. At below 50 mM NaCl, KCl, and NH_4Cl, the activity of intermedilysin was completely lost after the incubation. However, 80 and 100%, 44 and 62%, 10 and 68%, 81 and 98% of the activity remained after incubation in the presence of 300 and 1,000 mM NaCl, KCl, NH_4Cl, and $(NH_4)_2SO_4$, respectively. Thus, among the chloride salts tested in this study, the order of stabilization affected by cations was $Na^+ > K^+ > NH_4^+$. Anion species also seemed to affect the stability of the toxin, because 300 mM ammonium sulfate (ideal ionic strength, 0.9) was more effective than 1 M ammonium chloride (ideal ionic strength, 1.0).

Effects of thiol-reactive reagents, cholesterol, and anti-streptolysin O antibody on intermedilysin activity. We compared the characteristics of intermedilysin with those of thiol-activated cytotoxins.

Typical thiol-activated cytotoxins are susceptible to reagents

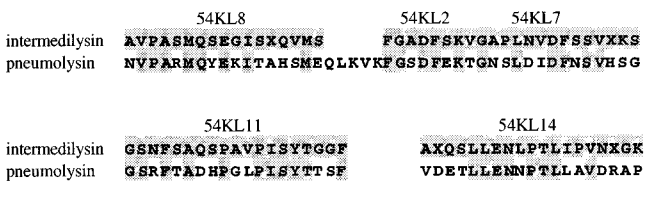

FIG. 4. Alignment of partial primary sequences of intermedilysin and pneumolysin. Identical amino acids are indicated by the shaded background.

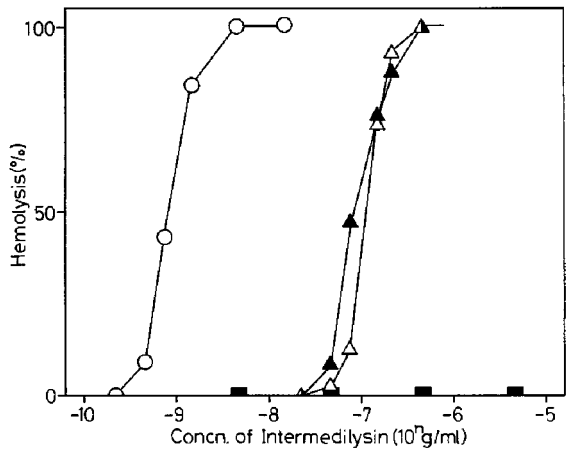

FIG. 5. Species specificity of hemolytic action by intermedilysin. Erythrocytes from various animals were used as target cells in the standard hemolysis assay as described in Materials and Methods. Open circles, human; unfilled triangles, chimpanzee; filled triangles, cynomolgus monkey; filled squares, cat, chicken, cow, dog, horse, mouse, rabbit, rat, and sheep. The results of one of five experiments using human, chimpanzee, and cynomolgus monkey samples and one of two experiments using erythrocytes from other animals are shown.

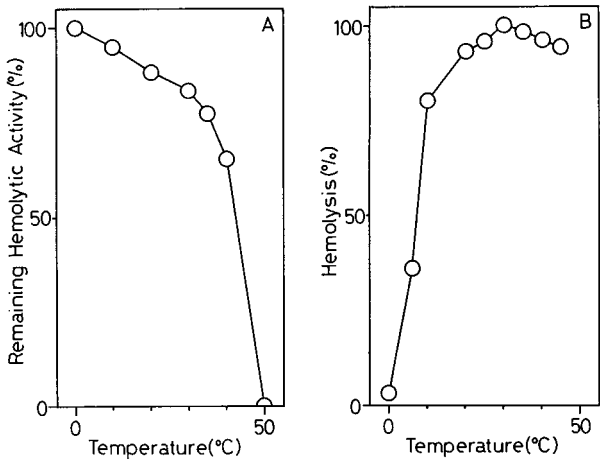

FIG. 6. Temperature stability profile and temperature dependence of the hemolytic action of intermedilysin. (A) Temperature stability profile of intermedilysin. To determine the stability of intermedilysin activity at various temperatures, 200 μl of PBS containing 7.3 ng of intermedilysin was incubated at 0, 10, 20, 25, 30, 35, 40, and 50°C for 1 h. Thereafter, microcentrifuge tubes containing the mixture were chilled once in an ice-water bath. Chilled PBS (800 μl) containing 5.0×10^7 human erythrocytes was mixed with intermedilysin and reacted at 37°C for 1 h. (B) Temperature dependence of the hemolytic action of intermedilysin. To investigate the temperature dependence of the hemolytic action of intermedilysin, standard hemolysis reaction mixtures containing human erythrocytes and 7.3 ng of intermedilysin were prepared at 0°C in an ice-water bath and then incubated at 0, 6, 10, 20, 25, 30, 35, 40, and 45°C for 1 h. The hemolysis induced purely by temperature was negligible, judging from the results of the hemolysis assay without intermedilysin over the same temperature range. The results of one of two experiments are shown.

that modify thiol residues, such as *N*-ethylmaleimide (NEM), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), and cholesterol (10, 15–17, 34) and are activated by reducing reagents such as DTT and β-mercaptoethanol (10, 15–17, 34, 39). Streptolysin O was activated about 30-fold (0.033-fold decrease of HD_{50}). However, DTT only slightly inhibited the intermedilysin activity in the same assay system (1.1-fold increment of HD_{50}) (Table 2). Moreover, NEM or DTNB did not dramatically inactivate intermedilysin. These reagents increased the HD_{50} at most 2.1-fold over that estimated in the absence of the chemical modifier. In contrast, the hemolytic activity of intermedilysin as well as that of an other thiol-activated cytotoxin, streptolysin O, was significantly inhibited by cholesterol (Table 3). However, the apparent half-maximal inhibitory concentration of cholesterol for the hemolytic action of intermedilysin was roughly 2 orders higher than that of streptolysin O in this assay. Although the partial homology of primary sequence between intermedilysin and thiol-activated cytotoxins was revealed, anti-streptolysin O antibody failed to neutralize the hemolytic activity of intermedilysin (Table 3).

TABLE 2. Effect of thiol-reducing, -oxidizing, and -alkylating reagents on intermedilysin activity

Cytotoxin	Reagent	$_wHD_{50}/_{w/o}HD_{50}$[a]
Intermedilysin	DTT, 10 mM	1.1
	NEM, 10 mM	1.1
	DTNB, 1 mM	2.1
Streptolysin O	DTT, 10 mM	0.033

[a] Average of duplicate determinations. HD_{50} was estimated in the following hemolysis assay. Various amounts of intermedilysin were incubated in the presence or absence of DTT, NEM, or DTNB in 200 μl of PBS at 37°C for 1 h. The activation of streptolysin O (catalog no. S5265; Sigma, St. Louis, Mo.) by DTT was simultaneously determined. To subtract the effect of the incubation at 37°C for 1 h and the solvents of reagents (i.e., ethanol for NEM and dimethyl sulfoxide for DTNB), reagent blank control experiments were also carried out. Thereafter, each sample was mixed with 800 μl of PBS containing 5×10^7 human erythrocytes and allowed to react at 37°C for 1 h. None of these reagents had significant hemolytic action on the erythrocytes. The HD_{50}s (medians of two determinations and minimum and maximum range) of intermedilysin and streptolysin O preparations without any treatment were 0.8 ± 0.2 ng and 3.2 ± 0.4 μl per 200 μl of the reconstituted solution, respectively. $_wHD_{50}$ and $_{w/o}HD_{50}$ are the HD_{50} values obtained in the presence and absence of reagents, respectively.

TABLE 3. Effect of cholesterol and anti-streptolysin O antibody on hemolytic activity of intermedilysin and streptolysin O

Cytotoxin	Effector	Hemolysis (%)[a]
Intermedilysin	Cholesterol	
	3 μM	100
	10 μM	90.4
	20 μM	19.2
	50 μM	0
	Anti-streptolysin O	100
Streptolysin O	Cholesterol	
	30 nM	100
	100 nM	44.9
	300 nM	2.3
	1 μM	0
	Anti-streptolysin O	0.3

[a] Average of duplicate determinations. Various amounts of cholesterol were incubated with 4 U of intermedilysin or streptolysin O in 990 μl of PBS or PBS containing 10 mM DTT per tube at 37°C for 10 min, respectively. To subtract the effect of the incubation at 37°C in the presence of the solvent of cholesterol (ethanol), reagent blank control experiments were also carried out. Anti-streptolysin O (final 200-fold dilution of original antiserum) was incubated with each toxin at 0°C for 1 h as described above. Thereafter, each sample was mixed with 10 μl of PBS containing 5×10^7 human erythrocytes and allowed to react at 37°C for 1 h. No significant hemolytic action by ethanol was found on the erythrocytes. U, the activity showing 50% hemolysis in the hemolysis assay in the absence (intermedilysin) or presence (streptolysin O) of 10 mM DTT.

Effect of trypsinization of the target cell surface on intermedilysin action. To examine whether any cell surface or integral membrane proteins participate in the intermedilysin action, we investigated the sensitivity of human erythrocytes to intermedilysin after trypsinization of the cell surface. As shown in Table 4, the digestion of human erythrocytes surface by trypsin significantly reduced the susceptibility of the cells to intermedilysin. This effect was caused not by trypsin inhibitor used as a scavenger of the remaining trypsin after the prote-

TABLE 4. Effect of digesting erythrocytes with trypsin on intermedilysin action

Expt	1st treatment	2nd treatment	Hemolysis (%)[a]
1	Nil	Nil	100.8 ± 3.1 (4)
2	Nil	Egg white trypsin inhibitor	102.9 ± 4.3 (4)
3	Nil	Trypsin-trypsin inhibitor complex	98.7 ± 4.8 (4)
4	Trypsin	Egg white trypsin inhibitor	10.3 ± 3.1 (4)[b]

[a] Mean ± standard deviation (*n*). Erythrocytes (10^9) were incubated with or without of 0.2 mg of *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (T-8642; Sigma) in 210 μl of PBS at 37°C for 1 h. The cells were recovered by centrifugation and reacted with 100 μl of a final concentration of 2 mg of trypsin inhibitor from chicken egg white (catalog no. 109878; Boehringer Mannheim, Mannheim, Germany) per ml in 1% (wt/vol) BSA in PBS at 25°C for 30 min. For another control experiment except for the control without treatments with protease and its inhibitor, erythrocytes were incubated in the second step with trypsin-trypsin inhibitor 0.2 mg: 0.2 mg at mixing complex which was formed by incubation in 100 μl of 1% BSA–PBS at 25°C for 30 min. The supernatants were removed after the centrifugation, and the erythrocytes were suspended in 1% BSA–PBS to make a 50% suspension for use in the assay of hemolysis induced by 6.7 ng of intermedilysin.

[b] Significant difference between experiment 4 and the others by Student's unpaired *t* test ($P < 0.001$).

olysis step or by trypsin-trypsin inhibitor complex but by digestion by trypsin.

DISCUSSION

We described a cytotoxic factor expressed by *S. intermedius* that causes blebs in human cells and ultimately cell death. We named this factor intermedilysin and suggest that it may contribute significantly to the pathogenicity exhibited by this bacterium.

During purification, most of the activity was lost after ion-exchange chromatography, but considerable activity remained after lyophilization of the culture supernatant. This may be due to the stabilization of intermedilysin at high salt concentrations. Therefore, hydrophobic chromatography after gel filtration was applied, which resulted in purification of the toxin. Major and minor bands of 54 and 53 kDa respectively, were found in SDS-PAGE analysis of purified intermedilysin. Immunoblotting using an antibody against peptide 54KL11 demonstrated that the bands shared a common protein structure but seemed to have microheterogeneity due to a posttranslational modification. The peak of cytolytic activity recovered by gel filtration had a molecular mass of about 50 kDa. Thus, intermedilysin seems to be a monomer under physiological, aqueous conditions. Internal partial amino acid sequencing of five fragments of intermedilysin revealed some homology with thiol-activated cytotoxins such as pneumolysin and streptolysin O, but the failure of anti-streptolysin O in the neutralization of intermedilysin suggests a difference in their steric structures. Moreover, several key features of intermedilysin differed from those of thiol-activated cytotoxins.

Intermedilysin was not activated by DTT, in contrast with streptolysin O, as shown in Table 2. Moreover, neither NEM nor DTNB drastically affected the cytolytic activity of intermedilysin. In general, the cysteine residue involved in the conserved sequence near the C terminus of thiol-activated cytotoxins can be substituted with small amino acids such as glycine or alanine without a fatal loss of hemolytic activity (5). However, chemical modification of the cysteine residue (10, 15–17, 34, 39) has indicated that the integrity of steric structure around the residue within wild-type thiol-activated cytotoxins is essential for maintaining the functional structure required for the cytolytic action. For example, thiol group modifiers such as 1 mM NEM and 0.5 mM DTNB cause a remarkable increment in the HD_{50} of perfringolysin O (68- and 34-fold by NEM and DTNB, respectively, compared with the HD_{50} in the absence of the modifier [15]). It is not yet known whether this difference in susceptibility between intermedilysin and thiol-activated cytotoxins is due to reactivities with the reagents or to structural changes induced by chemical modification. However, these results indicated that there is a critical structural difference between intermedilysin and typical thiol-activated cytotoxins.

The most distinctive property of intermedilysin was its species specificity. Figure 5 shows that human erythrocytes were highly sensitive to the toxin (HD_{50} of intermedilysin was 0.8 ng [=15 fmol]). This value is equivalent to 178 molecules of intermedilysin per erythrocyte, which is sufficient to form pores in the cell membrane as seen with pneumolysin (24). On the contrary, erythrocytes of two primates (chimpanzee, one of the species most closely related to humans, and cynomolgus monkey, an Old World monkey that separated from the ancestor of humans over 20 million years ago [32]) were both 2 orders less susceptible to intermedilysin than those of humans. Other animal erythrocytes were actually resistant to the toxin. Human-specific cytolytic action is unique and has never been identified in other cytolytic toxins (1).

In general, the first step of cytolysis is thought to be the binding of toxin to the cell surface target molecule (receptor) corresponding to each cytotoxin. Some common molecules such as cholesterol and glycochains on glycolipids are recognized as receptors (7, 34, 46). Since cholesterol inhibited the action of intermedilysin, the interaction between the toxin and cholesterol in the cell membrane may participate in the overall cytolytic reaction by intermedilysin. However, the significant human specificity of intermedilysin, the requirement for large amounts of cholesterol to inhibit its action, and its inhibition after digesting the target cell surface with trypsin are not explainable by only the interaction between the toxin and cholesterol. A novel protein or intramolecular structure of a known cell membrane protein which is human specific, rather than a generic molecule present in all animal cells, probably contributes to the species specificity of the toxin. The species specificity probably depends on the existence and/or structure of the key molecule, for example, the receptor for intermedilysin or the interactive protein with the toxin in the postadsorption step to the cells, which is of interest from the aspect of not only the pathogenicity shown by *S. intermedius* in humans but also the molecular evolution of the key molecule itself. Judging from the increase in susceptibility to intermedilysin shown in Fig. 5, such a molecular change may have accompanied the evolution of primates, especially the separation of humans from other primates that occurred 5 million years ago (32).

In this study, intermedilysin lysed human nucleated cells (e.g., NB69 [brain] and HepG2 [liver] cells) established from sites that were preferentially invaded by *S. intermedius*. The preliminary experiments showed that other human cell lines from various organs were also susceptible to the toxin, although to a lesser degree than the cell lines shown in Fig. 1 (24a). Thus, further systematic study of the susceptibility of a series of human cells established from various organs to intermedilysin is of interest to determine whether the toxin is involved in the infection tropism of this bacterium. Moreover, the expression of intermedilysin analogs by the genetically related species *S. anginosus* and *S. constellatus* should be analyzed from the viewpoint of the characterization of pathogenicity exhibited by each species of the SMG.

To evaluate the contribution of intermedilysin to the pathogenicity of *S. intermedius* and to understand the human-specific action shown by the toxin in the context of the evolution of the host-parasite interaction, the complete structure and cytolytic mechanism of intermedilysin should be determined as the next step.

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REFERENCES

1. **Alouf, J. E.** 1976. Cell membranes and cytolytic bacterial toxins, p. 220–270. *In* P. Cuatrecasas (ed.), The specificity and action of animal, bacterial and plant toxins. receptors and recognition series B, vol. 1. Chapman & Hall, London.
2. **Arala-Chaves, M. P., T. B. Higerd, M. T. Porto, J. Munoz, J.-M. Goust, H. H. Fudenberg, and C. B. Loadholt.** 1979. Evidence for the synthesis and release of strongly immunosuppressive, noncytotoxic substances by *Streptococcus intermedius*. J. Clin. Invest. **64:**871–883.
3. **Bateman, N. T., S. J. Eykyn, and I. Phillips.** 1975. Pyogenic liver abscess caused by *Streptococcus milleri*. Lancet **i:**657–659.
4. **Beighton, D., and R. A. Whiley.** 1990. Sialidase activity of the "*Streptococcus milleri* group" and other viridans group streptococci. J. Clin. Microbiol. **28:**1431–1433.

5. **Braun, V., and T. Focareta.** 1991. Pore-forming bacterial protein hemolysins (cytolysins). Crit. Rev. Microbiol. **18:**115–158.
6. **Brook, I., and E. H. Frazier.** 1994. Microaerophilic streptococci as a significant pathogen: a twelve-year review. J. Med. **25:**129–144.
7. **Cabiaux, V., P. Lorge, M. Vandenbranden, P. Falmagne, and J. M. Ruysschaert.** 1985. Tetanus toxin induces fusion and aggregation of lipid vesicles containing phosphatidylinositol at low pH. Biochem. Biophys. Res. Commun. **128:**840–849.
8. **Chua, D., H. H. Reinhart, and J. D. Sobel.** 1989. Liver abscess caused by *Streptococcus milleri*. Rev. Infect. Dis. **11:**197–202.
9. **De Louvois, J.** 1980. Bacteriological examination of pus from abscesses of the central nervous system. J. Clin. Pathol. **33:**66–71.
10. **Geoffroy, C., and J. E. Alouf.** 1983. Selective purification by thiol-disulfide interchange chromatography of alveolysin, a sulfhydryl-activated toxin of *Bacillus alvei.* J. Biol. Chem. **258:**9968–9972.
11. **Geoffroy, C., J. Mengaud, J. E. Alouf, and P. Cossart.** 1990. Alveolysin, the thiol-activated toxin of *Bacillus alvei*, is homologous to listeriolysin O, perfringolysin O, pneumolysin, and streptolysin O and contains a single cysteine. J. Bacteriol. **172:**7301–7305.
12. **Gossling, J.** 1988. Occurrence and pathogenicity of the *Streptococcus milleri* group. Rev. Infect. Dis. **10:**257–285.
13. **Homer, K. A., M. C. Grootveld, J. Hawkes, D. P. Naughton, and D. Beighton.** 1994. Degradation of hyaluronate by *Streptococcus intermedius* strain UNS35. J. Med. Microbiol. **41:**414–422.
14. **Homer, K. A., R. A. Whiley, and D. Beighton.** 1994. Production of specific glycosidase activities by *Streptococcus intermedius* strain UNS35 grown in the presence of mucin. J. Med. Microbiol. **41:**184–190.
15. **Iwamoto, M., Y. Ohno-Iwashita, and S. Ando.** 1987. Role of the essential thiol group in the thiol-activated cytolysin from *Clostridium perfringens*. Eur. J. Biochem. **167:**425–430.
16. **Jacobs, A. A. C., P. L. W. Loeffen, A. J. G. Van den Berg, and P. K. Storm.** 1994. Identification, purification, and characterization of a thiol-activated hemolysin (Suilysin) of *Streptococcus suis*. Infect. Immun. **62:**1742–1748.
17. **Johnson, M. K.** 1972. Properties of purified pneumonococcal hemolysin. Infect. Immun. **6:**755–760.
18. **Kehoe, M. A., L. Miller, J. A. Walker, and G. J. Boulnois.** 1987. Nucleotide sequence of the streptolysin O (SLO) gene: structural homologies between SLO and other membrane-damaging, thiol-activated toxins. Infect. Immun. **55:**3228–3232.
19. **Koepke, J. A.** 1965. Meningitis due to *Streptococcus anginosus* (Lancefield group F). JAMA **193:**739–740.
20. **Kyhse-Andersen, J.** 1984. Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J. Biochem. Biophys. Methods **10:**203–209.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
22. **Lütticken, R., U. Wendorff, D. Lütticken, E. A. Johnson, and L. W. Wannamaker.** 1978. Studies on streptococci resembling *Streptococcus milleri* and on an associated surface-protein antigen. J. Med. Microbiol. **11:**419–431.
23. **Mengaud, J., M.-F. Vicente, J. Chenevert, J. M. Pereira, C. Geoffroy, B. Gicquel-Sanzey, F. Baquero, J.-C. Perez-Diaz, and P. Cossart.** 1988. Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*. Infect. Immun. **56:**766–772.
24. **Morgan, P. J., S. C. Hyman, O. Byron, P. W. Andrew, T. J. Mitchell, and A. J. Rowe.** 1994. Modeling the bacterial protein toxin, pneumolysin, in its monomeric and oligomeric form. J. Biol. Chem. **269:**25315–25320.

24a. **Nagamune, H., et al.** Unpublished observations.

25. **Nagamune, H., K. Muramatsu, T. Akamatsu, Y. Tamai, K. Izumi, A. Tsuji, and Y. Matsuda.** 1995. Distribution of the Kexin family proteases in pancreatic islets: PACE4C is specifically expressed in B cells of pancreatic islets. Endocrinol. **136:**357–360.
26. **Ottens, H., and K. C. Winkler.** 1962. Indifferent and haemolytic streptococci possessing group-antigen F. J. Gen. Microbiol. **28:**181–191.
27. **Parker, M. T., and L. C. Ball.** 1976. Streptococci and aerococci associated with systemic infection in man. J. Med. Microbiol. **9:**275–302.
28. **Poole, P. M., and G. Wilson.** 1977. *Streptococcus milleri* in the appendix. J. Clin. Pathol. **30:**937–942.
29. **Posnett, D. N., H. McGrath, and J. P. Tam.** 1988. A novel method for producing anti-peptide antibodies. J. Biol. Chem. **263:**1719–1725.
30. **Ruoff, K. L.** 1988. *Streptococcus anginosus* ("*Streptococcus milleri*"). Clin. Microbiol. Newsl. **10:**65–68.
31. **Ruoff, K. L.** 1988. *Streptococcus anginosus* ("*Streptococcus milleri*"): the unrecognized pathogen. Clin. Microbiol. Rev. **1:**102–108.
32. **Saitou, N., and S. Ueda.** 1994. Evolutionary rates of insertion and deletion in noncoding nucleotide sequences of primates. Mol. Biol. Evol. **11:**504–512.
33. **Singh, K. P., A. Morris, S. D. R. Lang, D. M. MacCulloch, and D. A. Bremner.** 1988. Clinically significant *Streptococcus anginosus* (*Streptococcus milleri*) infections: a review of 186 cases. N. Z. Med. J. **101:**813–816.
34. **Smyth, C. J., and J. L. Duncan.** 1978. Thiol-activated (oxygen-labile) cytolysins, p. 129–183 *In* J. Jeljaszewicz and T. Wadstrom (ed.), Bacterial toxins and cell membranes. Academic Press, London.
35. **Taketoshi, M., K. Kitada, T. Yakushiji, and M. Inoue.** 1993. Enzymatic differentiation and biochemical and serological characteristics of the clinical isolates of *Streptococcus anginosus*, *S. intermedius* and *S. constellatus*, Microbios **76:**115–129.
36. **Terleckyj, B., N. P. Willett, and G. D. Shockman.** 1975. Growth of several cariogenic strains of oral streptococci in a chemically defined medium. Infect. Immun. **11:**649–655.
37. **Tweten, R. K.** 1988. Nucleotide sequence of the gene for perfringolysin O (theta-toxin) from *Clostridium perfringens*: significant homology with genes for streptolysin O and pneumolysin. Infect. Immun. **56:**3235–3240.
38. **Van der Auwera, P.** 1985. Clinical significance of *Streptococcus milleri*. Eur. J. Clin. Microbiol. **4:**386–390.
39. **Van Epps, D. E., and B. R. Andersen.** 1971. Streptolysin O. II. Relationship of sulfhydryl groups to activity. Infect. Immun. **3:**648–652.
40. **Walker, J. A., R. L. Allen, P. Falmagne, M. K. Johnson, and G. J. Boulnois.** 1987. Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae*. Infect. Immun. **55:**1184–1189.
41. **Whiley, R. A., and D. Beighton.** 1991. Emended descriptions and recognition of *Streptococcus constellatus*, *Streptococcus intermedius*, and *Streptococcus anginosus* as distinct species. Int. J. Syst. Bacteriol. **41:**1–5.
42. **Whiley, R. A., D. Beighton, T. G. Winstanley, H. Y. Fraser, and J. M. Hardie.** 1992. *Streptococcus intermedius*, *Streptococcus constellatus*, and *Streptococcus anginosus* (the *Streptococcus milleri* group): association with different body sites and clinical infections. J. Clin. Microbiol. **30:**243–244.
43. **Whiley, R. A., H. Fraser, J. M. Hardie, and D. Beighton.** 1990. Phenotypic differentiation of *Streptococcus intermedius*, *Streptococcus constellatus*, and *Streptococcus anginosus* strains within the "*Streptococcus milleri* group." J. Clin. Microbiol. **28:**1497–1501.
44. **Whiley, R. A., and J. M. Hardie.** 1989. DNA-DNA hybridization studies and phenotypic characteristics of strains within the '*Streptococcus milleri* group.' J. Gen. Microbiol. **135:**2623–2633.
45. **Willcox, M. D. P., M. Patrikakis, C. Y. Loo, and K. W. Knox.** 1993. Albumin-binding proteins on the surface of the *Streptococcus milleri* group and characterization of the albumin receptor of *Streptococcus intermedius* C5. J. Gen. Microbiol. **139:**2451–2458.
46. **Wisnieski, B. J., and J. S. Bramhall.** 1981. Photolabelling of cholera toxin subunits during membrane penetration. Nature (London) **289:**319–321.
47. **Yamaguchi, T., M. Taketoshi, H. Eifuku-Koreeda, T. Yakushiji, and M. Inoue.** 1993. Haemagglutinating activities of oral strains of *Streptococcus milleri* group. Microbios **75:**249–259.

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